Bone Sialoprotein Mediates the Tumor Cell–Targeted Prometastatic Activity of Transforming Growth Factor β in a Mouse Model of Breast Cancer

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Abstract

Transforming growth factor βs (TGF-β) play a dual role in carcinogenesis, functioning as tumor suppressors early in the process, and then switching to act as prometastatic factors in late-stage disease. We have previously shown that high molecular weight TGF-β antagonists can suppress metastasis without the predicted toxicities. To address the underlying mechanisms, we have used the 4T1 syngeneic mouse model of metastatic breast cancer. Treatment of mice with a monoclonal anti-TGF-β antibody (1D11) significantly suppressed metastasis of 4T1 cells to the lungs. When metastatic 4T1 cells were recovered from lungs of 1D11-treated and control mice, the most differentially expressed gene was found to be *bone sialoprotein* (*Bsp*). Immunostaining confirmed the loss of Bsp protein in 1D11-treated lung metastases, and TGF-β was shown to regulate and correlate with Bsp expression *in vitro*. Functionally, knockdown of Bsp in 4T1 cells reduced the ability of TGF-β to induce local collagen degradation and invasion *in vitro*, and treatment with recombinant Bsp protected 4T1 cells from complement-mediated lysis. Finally, suppression of Bsp in 4T1 cells reduced metastasis *in vivo*. We conclude that Bsp is a plausible mediator of at least some of the tumor cell–targeted prometastatic activity of TGF-β in this model and that Bsp expression in metastases can be successfully suppressed by systemic treatment with anti-TGF-β antibodies. (Cancer Res 2006; 66(12): 6327-35)

Introduction

The transforming growth factor βs (TGF-β) are multifunctional growth factors that play particularly complex roles in tumorigenesis. Clinical and mouse model data show that the TGF-β pathway clearly has tumor suppressor activity and reduction or loss of TGF-β receptors or downstream signaling components is seen in many human tumors (reviewed in ref. 1). However, late-stage human tumors frequently show a paradoxical increase in expression of TGF-βs that is associated with metastasis and poor prognosis (2). The unifying hypothesis is that TGF-βs have tumor suppressor activity early in the carcinogenic process, but that in the later stages, suppressor activity is lost and pro-oncogenic activities prevail (1, 3).

The dual role for TGF-β in carcinogenesis poses a major therapeutic challenge, as strategies must be sought to specifically target the prometastatic activities of TGF-βs while sparing the desirable effects on normal homeostasis and tumor suppression. We have previously shown that prolonged exposure to a high molecular weight TGF-β antagonist of the receptor:Fc fusion protein class could suppress metastasis without significant side effects in a mouse model system, raising the possibility that this goal might be achievable under certain circumstances (4). Other studies have also provided evidence that targeting the TGF-β pathway could be a powerful approach to the treatment or prevention of metastasis (5–9).

Successful application of TGF-β antagonists to prevent or suppress metastatic disease will depend on the ability to stratify patients in such a way as to exclude those who might show an adverse response to treatment. In addition, it will be useful to have molecular biomarkers that correlate with response to antagonist treatment. We believe that a better understanding of mechanisms underlying both the tumor suppressor and prometastatic effects of TGF-β *in vivo* will be critical for the achievement of these goals.

A large body of experimental data has suggested that TGF-β has the potential to promote metastasis through effects both on the tumor cell itself and on other cellular compartments. Direct effects on the tumor cell that might promote metastasis include induction of an epithelial-to-mesenchymal transition, promotion of migration and invasion, and enhanced tumor cell survival (reviewed in ref. 1). By contrast, indirect effects of TGF-β that could increase metastatic efficiency include its ability to suppress the immune surveillance system and to promote angiogenesis (reviewed in ref. 1). While the existing experimental data have been helpful in establishing the spectrum of possible activities of TGF-β *in vivo*, it is currently less clear which of these various activities are actually engaged by a developing tumor and, more specifically, which activities might be accessible to modulation by TGF-β antagonists.

Here we have used the 4T1 mouse model of metastatic breast cancer to investigate mechanisms underlying metastasis suppression when the TGF-β system is antagonized using an anti-TGF-β monoclonal antibody (1D11) that recognizes all three isoforms of TGF-β. The 4T1 model, which is syngeneic to BALB/c mice, is widely considered to be one of the best models of postoperative stage IV breast cancer, and the 4T1 cells can metastasize to lung, liver, and bone following tail-vein injection or orthotopic implantation (10, 11). In this study, we have focused on the possible effects of TGF-β antagonism on the tumor cells themselves. We present

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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evidence that bone sialoprotein (Bsp) may be a key mediator of the prometastatic effects of TGF-β in this model of breast cancer and that anti-TGF-β antibodies may act, in part, through the down-regulation of Bsp in the metastatic cells.

Materials and Methods

Cell culture and reagents. 67NR, 4TO7, and 4T1 cell lines were provided by Dr. Fred Miller (Barbara Ann Karmanos Cancer Institute, Detroit, MI) and were cultured as previously described (10). TGF-β1 and TGF-β type I receptor kinase inhibitor (ALK5 inhibitor I) were purchased from R&D Systems (Minneapolis, MN) and Calbiochem (La Jolla, CA), respectively. The anti-TGF-β murine monoclonal antibody, 1D11, which neutralizes all three isoforms of TGF-β (12), and an isotype-matched immunoglobulin G1 monoclonal antibody, 13C4, which was raised against Shigella toxin and serves as a control, were provided by Genzyme Corporation (Framingham, MA).

***In vivo* metastasis study.** All animals were maintained according to the National Cancer Institute Animal Care and Use Committee guidelines under approved animal study protocols. For the spontaneous metastasis format, the left thoracic (#2) mammary glands of anesthetized 7-week-old female BALB/cAnCr mice (National Cancer Institute-Frederick, Frederick, MD) were surgically exposed and 4×10^4 4T1 cells were inoculated into the mammary fat pad in a 40-μL volume. After inoculation, the mice were randomized into two treatment groups with 17 to 20 animals per group. Anti-TGF-β antibody (1D11, 5 mg/kg body weight) was administered thrice per week i.p., starting 1 day after cell inoculation. The control group received the same dosage and volume of the control monoclonal antibody 13C4. Primary tumors were surgically excised on day 10. Mice were euthanized by carbon dioxide narcosis on day 28 and the lungs were removed, inflated, and fixed in 10% buffered formalin. The relative lung weight was calculated using the formula (lung weight / body weight) × 100 (%). Macroscopic quantitation of metastases was done by counting the number of nodules on the surface of the lung. For microscopic quantitation of lung metastases, each lobe of the lung was processed for H&E staining and evaluated by a board-certified veterinary pathologist (Miriam R. Anver, D.V.M., Ph.D.). For the experimental metastasis format, 5,500 4T1 cells were injected into the tail-vein of 7-week-old female BALB/c mice. Lungs were harvested on day 21 and analyzed as above.

Recovery of metastatic cells from lungs. Lungs were harvested from tumor-bearing mice treated with 1D11 (anti-TGF-β) or 13C4 (control) antibodies, minced, and digested for 1 hour with 1 mg/mL type IV collagenase (Sigma-Aldrich, St. Louis, MO), suspended in DMEM (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS). After spinning out debris, the cell digests were placed in culture medium containing 10 μg/mL of 6-thioguanine (Sigma-Aldrich) for several days to enrich for 4T1 cells.

Oligonucleotide microarray analysis. RNA was prepared from five independent isolates of metastatic 4T1 cells recovered from the lungs of 1D11-treated and control mice using RNeasy Mini kit according to the instructions of the manufacturer (Qiagen, Valencia, CA). The Affymetrix Gene Chip MOE430A (Affymetrix, Santa Clara, CA) was used for analysis. cDNA synthesis and cRNA *in vitro* transcription, labeling, and linear amplification were done using the Two-cycle cDNA Synthesis Kit and GeneChip IVT Labeling Kit (Affymetrix). The *in vitro* transcription products were purified, fragmented, and hybridized to the oligonucleotide arrays as recommended by the manufacturer. Raw data were processed with Robust Multiarray Average algorithm and quantile normalization to obtain gene summary measures (13). Differences in gene expression levels between the two treatment groups were identified using univariate two-sample *t* test ($P < 0.001$). The statistical computations were done using the R and Affy package of the Bioconductor software project.[5]

Quantitative reverse transcription-PCR. To validate the microarray results, real-time quantitative PCR was done with the iCycler iQ Real-time PCR Detection System (Bio-Rad, Hercules, CA) using SYBR green dye (Stratagene, Cedar Creek, TX). First-strand cDNA was prepared from total RNA using a SuperScript III first strand synthesis kit (Invitrogen). The quantitative reverse transcription-PCR (RT-PCR) was done in triplicate. Mouse Bsp mRNA levels were normalized to mouse 28S rRNA. The primer sets used in this study were as follows: Bsp, 5′-TTCCCAGGTGTGTCATTGAAGA-3′ (forward primer) and 5′-GGTATGTTTGCGCAGTTAGCAA-3′ (reverse primer); and 28S rRNA, 5′-GGGTGGTAAACTCCATCTAA-3′ (forward primer) and 5′-AGTTCTTTTCAACTTTCCCT-3′ (reverse primer).

Immunoblotting, immunohistochemistry, and ELISA assays for Bsp and TGF-β1. Immunoblotting was done as previously described (14). Membranes were probed with anti-Bsp polyclonal antibody LF-84 (1:1,000 dilution; ref. 15) and anti-β-actin monoclonal antibody (clone AC-15, 1:5,000 dilution; Sigma-Aldrich). For immunostaining of formalin-fixed samples for Bsp, the avidin-biotin-peroxidase complex method was used with the anti-Bsp polyclonal antibody LF-84, as above, at a final dilution of 1:100. Lung metastases were individually evaluated for Bsp expression using a semiquantitative score system as follows: 0, no Bsp-positive 4T1 cells in the metastasis; 1, <30% positive cells; 2, 30% to 60% positive cells; and 3, >60% positive cells. Metastases were scored for three mice from each treatment group for a total of 152 metastases. The difference in score between the two treatment groups was assessed by the likelihood ratio test of the β binomial model, grouping metastases with a score of 0 and 1 or 2 and 3 for each mouse. Circulating Bsp levels in serum were determined using a competitive ELISA assay following separation of Bsp from complement Factor H by denaturation, reduction, and column chromatography, as described (16). Total TGF-β1 levels in clarified serum-free cell-conditioned medium were determined without sample acidification using Quantikine TGF-β1 ELISA kit (R&D Systems) according to the instructions of the manufacturer.

Promoter-reporter assays. 4T1 cells at 50% confluence in 12-well plates were transfected with a total of 100 ng/well of promoter-reporter plasmid, together with 10 ng/well of pRL-TK (*Renilla*) plasmid DNA for normalization, using Fugene 6 transfection reagent (Roche, Indianapolis, IN) according to the instructions of the manufacturer. The promoter-reporter constructs used were $pCAGA_{12}LUC$, containing 12 repeats of the Smad3 binding element $CAGA_{12}$ driving luciferase (17), or p1.4 kbBsp-LUC, with the proximal 1.4 kb of the mouse *Bsp* promoter driving luciferase (18). After transfection, cells were incubated for 16 to 18 hours in the presence or absence of TGF-β1 (5 ng/mL) and cell lysates were harvested for assay using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). Luminescence was measured by VICTOR^2 (Perkin-Elmer Life and Analytical Sciences, Boston, MA).

Transfection of cells with small interfering RNA. Four small interfering RNA (siRNA) sequences were chosen to target mouse *Bsp* gene (GeneBank accession no. NM_008318) according to the Dharmacon siGENOME protocol (Dharmacon, Lafayette, CO). The positions of the target sequences for these four siRNA duplexes, designated as siB1, siB2, siB3, and siB4, are as follows: siB1, bases 203 to 221; siB2, bases 1,023 to 1,041; siB3, bases 975 to 993; and siB4, bases 909 to 927 in the nucleotide sequence of *Bsp*. Green fluorescent protein siRNA (green fluorescent protein duplex I, Dharmacon) was used as a nonspecific siRNA control. siRNA was introduced into the cells by reverse transfection using Silentfect lipid reagent (Bio-Rad). Briefly, serum-free DMEM containing Silenfect lipid reagent (Bio-Rad) was added to the culture flask. Next, serum-free DMEM containing siRNA was added. The lipid/siRNA mixture was incubated for 30 minutes at ambient temperature. 4T1 cells were trypsinized and washed thrice with DMEM. Cells were then resuspended in DMEM supplemented with 10% FBS. Cell suspension was added to the lipid/siRNA mixture and incubated for 45 minutes at ambient temperature followed by incubation at 37°C (CO_2, humidified). The final siRNA concentration was 50 nmol/L.

Complement-mediated cell lysis assay. The susceptibility of 4T1 cells to complement-mediated cell lysis was determined as previously described (19). Briefly, trypsinized cells were preincubated with 10 μg/mL of purified human Bsp protein for 10 minutes at 37°C, followed by addition of normal human serum to a final dilution of 1:5 to 1:20. After an additional 2-hour incubation at 37°C, cell viability was evaluated using a colorimetric MTT assay (Chemicon, Temecula, CA).

[5] http://www.bioconductor.org.

Matrigel invasion assay. Breast cancer cell invasion was assayed in 24-well Biocoat Matrigel invasion chambers (8 μm; BD Biosciences, Bedford, MA) according to the protocol of the manufacturer. Briefly, the top chamber was seeded with 1×10^5 viable tumor cells in serum-free medium (AIM-V medium, Invitrogen). The bottom chamber was filled with DMEM supplemented with 10% FBS as a chemoattractant. After 20 hours of incubation, the noninvasive cells that remained on the upper surface of the membrane were removed with a cotton swab. Cells that had migrated through the membrane were then fixed with methanol and stained with hematoxylin (Vector, Burlingame, CA). Migratory cells per field were counted for three random fields for each membrane using a light microscope at ×200 magnification. Invasion assays were done in triplicate.

Conventional and *in situ* zymography. Conventional zymography of cell-free culture supernatants harvested from cells grown on type I collagen was done using 10% polyacrylamide, 0.1% gelatin gels as described (20). *In situ* zymography was also done as previously described (21). In brief, cells grown on glass coverslips were washed twice with PBS, overlaid with a solution of 50 μg/mL fluorescein-labeled collagen I or collagen IV (DQ collagen, Molecular Probes, Eugene, OR), 1% (w/v) low-melting agarose (BME, Rockland, ME), and 5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) in PBS, and incubated on ice for 15 minutes, followed by incubation in a humidified chamber at room temperature for 3 hours. Specimens were fixed in 10% buffered formalin (Sigma-Aldrich) and were examined by confocal microscopy (Leica DM IRBE confocal microscopy, ×630 magnification). Nuclei and collagen degradation were visualized as blue and green fluorescence, respectively. The calculation of collagen degradation is based on the green fluorescence area and density divided by the cell number, as determined from the number of DAPI-stained nuclei, in three randomly selected fields for each specimen from a total of three independent experiments, using the Image-Pro Plus program (MediaCybernetics, Silver Spring, MD). For the quantitation, an arbitrary threshold was set to distinguish specific from background staining, and this same threshold setting was applied to all the samples analyzed.

Statistical analysis. Unpaired parametric Student *t* test and nonparametric Mann-Whitney *U* test were used to analyze the data, unless otherwise indicated in the text.

Results

The 1D11 anti-TGF-β monoclonal antibody suppresses metastasis of 4T1 cells to the lung. First, we evaluated whether the anti-TGF-β antibody could regulate metastasis of 4T1 cells in syngeneic BALB/c mice, following orthotopic implantation into the mammary fat pad (Fig. 1*A*). When initiated 1 day after tumor cell implantation, 1D11 treatment significantly reduced metastasis to the lung as evidenced by the decreased relative lung weight (Fig. 1*B*) and the reduction (~2-fold) in number of macroscopically visible metastatic lung nodules in 1D11-treated mice as compared with the control group (Fig. 1*C*). Pathologic examination confirmed that the macroscopic nodules represented lung metastases (data not shown). Taken together, these results clearly show that the anti-TGF-β antibody can suppress metastasis of 4T1 cells to the lungs from the orthotopic site.

Metastatic cell cultures recovered from 1D11-treated lungs show altered morphology and gene expression patterns. Previous studies have shown that TGF-β can have prometastatic effects that target the tumor cell directly (6, 22). We hypothesized that part of the therapeutic efficacy of the TGF-β antibody might be due to changes in gene expression in the tumor cells themselves. Recovery of high-quality RNA from lung metastases by laser capture microdissection has proved to be a major technical challenge; thus, we instead decided to recover and briefly culture metastatic cells from treated and untreated lungs before assay. To do this, we digested the tumor-bearing lungs with collagenase and placed the resulting cells in culture under selection with

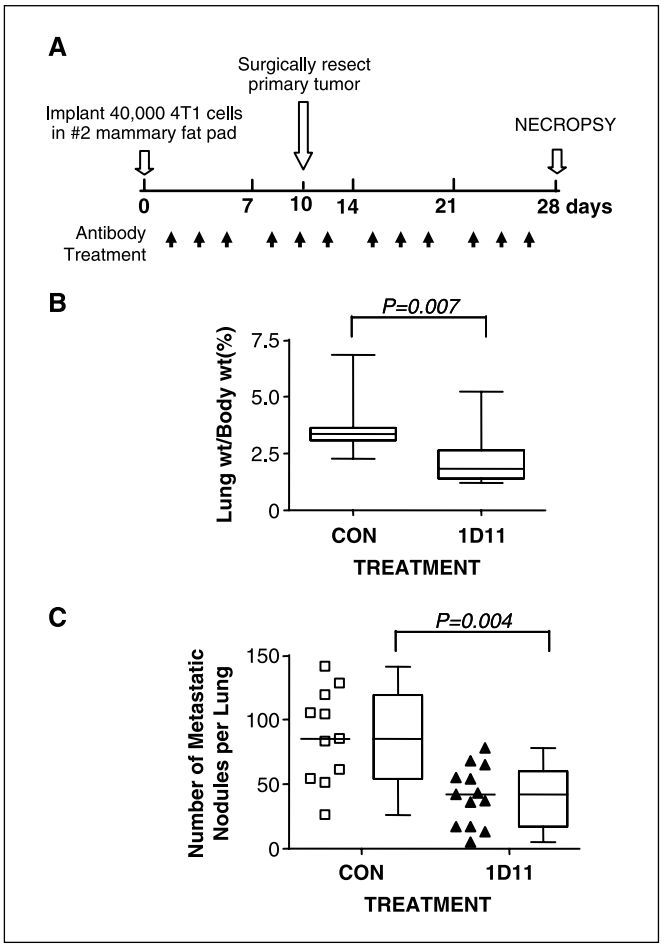

Figure 1. 1D11 decreases 4T1 metastasis to the lung following orthotopic implantation. *A,* experimental schema. 4T1 cells (4×10^4) were inoculated into the left thoracic mammary fat pad of BALB/c mice. After the inoculation, either anti-TGF-β antibody 1D11 (5 mg/kg) or control 13C4 antibody (*CON*; 5 mg/kg) was administered thrice per week i.p. to the mice. The primary tumors were removed on day 10 after the inoculation and mice were euthanized for necropsy on day 28. *B,* relative lung weight at necropsy. *C,* total number of grossly visible metastatic nodules in the lung. *Boxes,* median values with upper and lower quartiles; *whiskers,* range (control group, 11 mice; 1D11 group, 13 mice).

6-thioguanine to enrich for metastatic tumor cells, as the 4T1 cells are resistant to this drug (10). We observed morphologic differences between metastatic cell cultures derived from 1D11-treated or control antibody–treated lungs, which persisted for up to 10 days in culture. Metastatic 4T1 cells cultured from the lungs of mice treated with 1D11 appeared rounder and less spindled compared with their counterparts from animals treated with control antibody (Fig. 2*A*). In agreement with this observation, it has previously been shown that 4T1 cells treated with a murine TGF-β receptor:Fc fusion protein are less spindled *in vivo* when observed in a dorsal skin window assay (5).

Because the morphologic effects of anti-TGF-β antibody treatment *in vivo* persisted in the cultured cells for several days, we reasoned that gene expression changes induced by *in vivo* treatment might also persist. We therefore compared gene expression patterns of the metastatic cells from treated and untreated mice after 7 days in culture using the Affymetrix GeneChip MOE430A. Forty genes differed at the $P = 0.001$ level between cultures from 1D11-treated or control antibody–treated mice (Supplementary Table S1). Of these, the most differentially expressed gene was *bone sialoprotein* (*Bsp*),

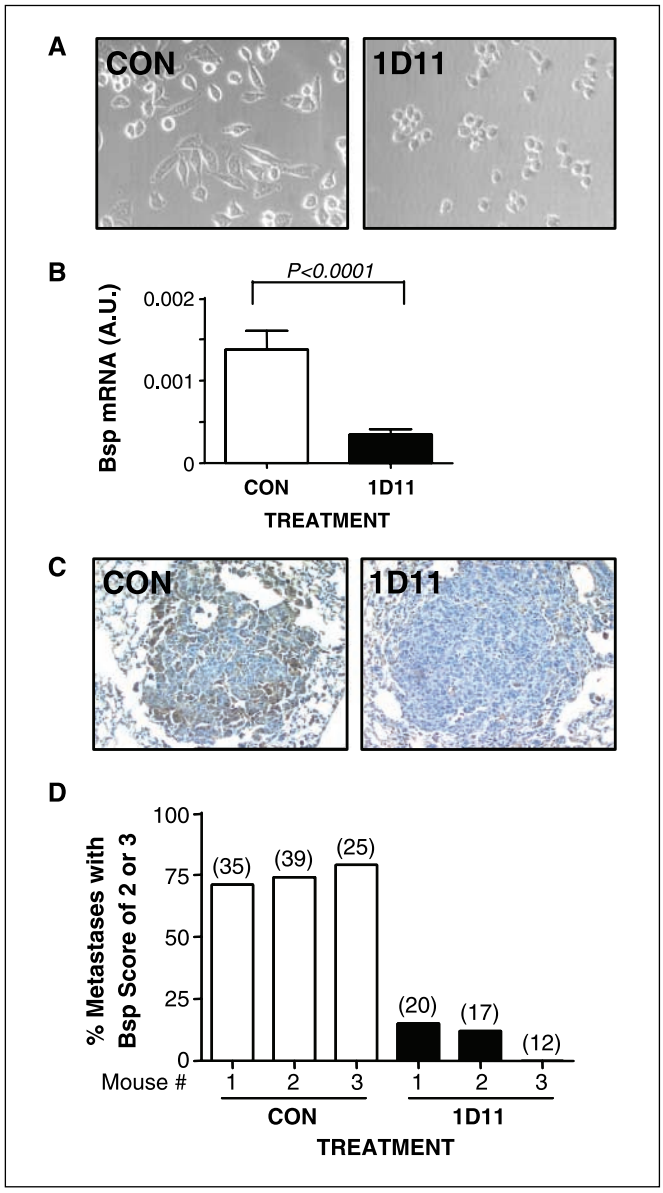

Figure 2. Metastatic cells recovered from 1D11-treated lungs show persistent morphologic changes in culture and elevated Bsp expression *in vitro* and *in vivo*. 4T1 cells were recovered from lungs harvested on day 28 as in Fig. 1 and cultured as described in Materials and Methods. *A,* morphologic appearance of the metastatic 4T1 cells cultured from the lungs of mice treated with anti-TGF-β (1D11) or control antibody after 7 days in culture. *B,* quantitative RT-PCR validation of differential expression of Bsp mRNA between metastatic cell cultures derived from treated and control mice. *Columns,* mean of five independent cell isolates per treatment group; *bars,* SD. Bsp mRNA expression was normalized to the 28S rRNA in each case. *C,* immunohistochemical staining for Bsp in lung metastases from treated and control mice. *D,* graphical representation of semiquantitative scoring for Bsp expression in lung metastases. Individual metastases were scored for Bsp expression on a scale of 0 to 3 as detailed in Materials and Methods. Percent metastases with a score of ≥2 for each of three mice per treatment group, for a total of 152 metastases evaluated. The number of metastases per mouse is indicated in parentheses above the columns.

also known as *integrin binding sialoprotein* (*Ibsp*), or *bone sialoprotein II* (*BspII*). Using quantitative RT-PCR, we confirmed a >3-fold reduction of Bsp mRNA level in metastatic 4T1 cells cultured from the lungs of mice treated with 1D11, as compared with their control counterparts (Fig. 2*B*). The other genes on the list had no known or proposed association with the metastatic process and were not pursued further.

Bsp expression is reduced in 1D11-treated metastases *in vivo*. Bsp is a member of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family of secreted integrin-binding proteins (23). It is overexpressed in many tumor types, including breast cancer, in which overexpression is associated with clinical severity and poor survival (24). Overexpression of Bsp in the human breast cancer cell line MDA-MB-231 has recently been shown to promote metastasis to bone and visceral organs (25). Thus, Bsp is a plausible candidate to mediate some of the prometastatic activity of TGF-β in breast cancer.

To investigate whether the observed decrease of Bsp mRNA expression in cultured metastatic cells reflected a loss of Bsp in the metastases *in vivo*, we immunostained lungs from treated and untreated mice. 1D11 treatment resulted in a substantial reduction in Bsp staining in the lung metastases when compared with the control counterparts (Fig. 2*C*). Bsp expression was scored semiquantitatively on a scale of 0 to 3 (see Materials and Methods) for a total of 152 metastases in six mice, and the fraction of metastases with a score of 2 or 3 is shown for each animal (Fig. 2*D*). The difference between the two treatment groups was highly significant.

Bsp expression is regulated by TGF-β and correlates with TGF-β expression and metastatic potential in cells *in vitro*. TGF-β regulates Bsp expression in a rat osteosarcoma cell line by transcriptional and posttranscriptional mechanisms (26). Here we showed that TGF-β treatment increased the expression of Bsp mRNA in 4T1 cells *in vitro* whereas treatment with a TGF-β receptor kinase inhibitor (ALK5 Inhibitor I) decreased it (Fig. 3*A*). The ability of the ALK5 Inhibitor I to reduce basal Bsp expression in the absence of added TGF-β suggests that there is a functional TGF-β autocrine loop operating in the 4T1 cells. However, TGF-β treatment did not up-regulate transcription from the *Bsp* promoter-reporter construct containing a previously described TGF-β response element, although it clearly enhanced transcription of a SMAD3-dependent $CAGA_{12}$-luciferase reporter construct (Fig. 3*B*). Accordingly, these results suggest that either TGF-β up-regulates Bsp in 4T1 cells *in vitro* exclusively through posttranscriptional mechanisms or any transcriptional response in the 4T1 cells involves a novel TGF-β regulatory element that is not present in the 1.4 kb of promoter sequence present in this construct.

We next examined Bsp and TGF-β expression in cell lines of increasing metastatic potential. 67NR, 4TO7, and 4T1 cell lines were derived from a single mammary tumor that arose spontaneously in a wild-type BALB/c mouse, and although they form primary tumors with similar kinetics, the cell lines differ dramatically in their metastatic potential (19). We observed a >100-fold increase of Bsp mRNA level in the 4TO7 cells, which can spread to but not colonize the lung, as compared with the nonmetastatic 67NR cells, which do not leave the primary site. Bsp expression was further increased by ~2-fold in the fully metastatic 4T1 cells (Fig. 3*C*). Interestingly, expression of total TGF-β1 protein showed a parallel increase (Fig. 3*C*). Taken together, these results clearly show that TGF-β and Bsp expressions correlate with each other, as well as with increasing metastatic ability, in this graded series of cell lines.

Bsp mediates TGF-β effects on invasion and collagen degradation *in vitro*. To address the possible importance of Bsp in mediating prometastatic activities of TGF-β, we developed siRNAs to reduce Bsp expression. We tested four siRNAs (siB1-siB4) designed from the mouse *Bsp* gene sequence. Overall, siB3 resulted in the greatest inhibition of Bsp mRNA expression (~3-fold) and was

selected for further study (Fig. 4*A*). Reduced Bsp expression persisted for at least 7 days in culture (data not shown). The expression of Bsp protein was also significantly suppressed by siB3, but not by control siRNA (Fig. 4*B*). Knockdown of Bsp had no effect on proliferation or survival of the 4T1 cells in culture (data not shown).

Bsp has been shown to promote the invasiveness of many cancer cell lines (27) and might therefore be a critical mediator of TGF-β-induced invasion. In a Matrigel invasion assay, addition of TGF-β caused a 1.5-fold increase in invasiveness of 4T1 cells. Knockdown of Bsp significantly reduced both basal and TGF-β-induced invasion to a similar level, suggesting that Bsp is necessary for the proinvasive activity of both autocrine and exogenously added TGF-β (Fig. 4*C*).

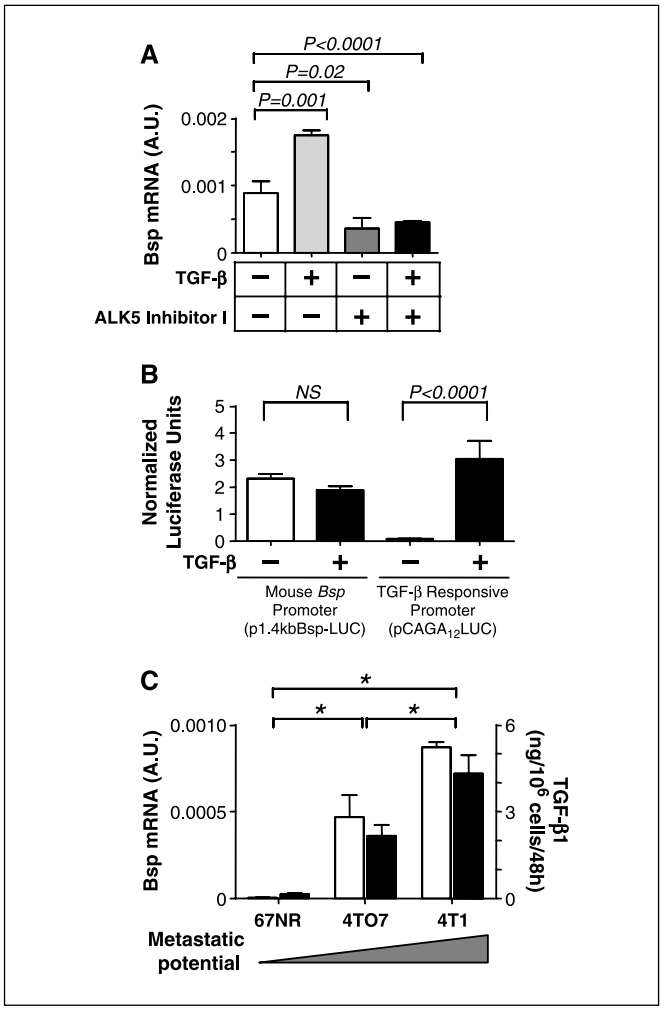

Figure 3. TGF-β regulates and is correlated with Bsp expression in 4T1-related cell lines *in vitro*. *A,* effect of TGF-β1 or a TGF-β type I receptor kinase inhibitor on the expression of Bsp mRNA by 4T1 cells *in vitro*. Cells were exposed to either TGF-β1 (5 ng/mL) or the ALK5 inhibitor I (10 μmol/L) for 48 hours before analyzing Bsp expression by quantitative RT-PCR. *B,* effect of TGF-β treatment on transcription from the mouse *Bsp* promoter was assessed by measuring luciferase activity in 4T1 cells transiently transfected with promoter-reporter constructs. p1.4 kbBsp-LUC is the mouse *Bsp* promoter-luciferase construct and pCAGA$_{12}$LUC is a synthetic TGF-β-responsive promoter-luciferase construct. Cells were treated with 5 ng/mL TGF-β1 for 18 hours before assay. *NS,* no significance. *C,* correlation between endogenous Bsp and TGF-β expression. Bsp mRNA expression (□) was determined by quantitative RT-PCR and secreted total (latent plus active) TGF-β1 protein levels (■) were determined by ELISA assay in a series of 4T1-related cells of increasing metastatic potential. *, $P < 0.001$. *Columns,* mean of three determinations; *bars,* SD.

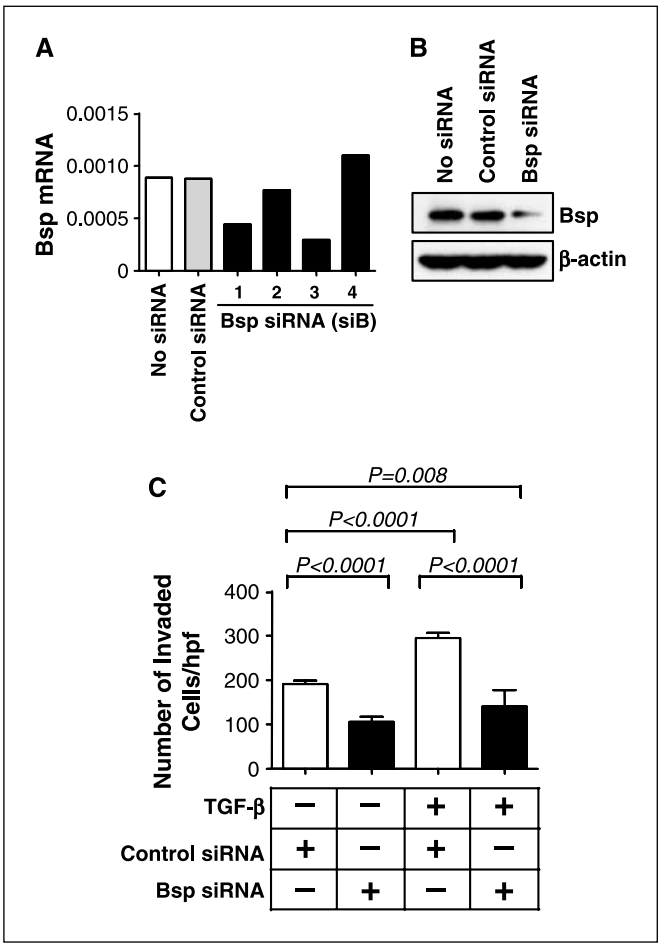

Figure 4. Bsp knockdown suppresses basal and TGF-β-induced invasion through Matrigel. *A,* quantitative RT-PCR analysis of Bsp expression following transfection of 4T1 cells with four different siRNA sequences targeting *Bsp*. An siRNA to green fluorescent protein was used as a negative control. *B,* Western blot analysis of Bsp protein levels in 4T1 cells 48 hours following transfection with the siB3 Bsp siRNA. *C,* effect of Bsp knockdown on invasiveness of 4T1 cells. 4T1 cells transfected with siB3 were assessed for their ability to invade through Matrigel using a Transwell assay system, with and without added TGF-β1 (5 ng/mL). *Columns,* mean of three determinations; *bars,* SD.

TGF-β is thought to increase the invasiveness and metastatic behavior of cancer cells, in part, by stimulating the degradation of extracellular matrix (28), and we did see a strong induction of matrix metalloproteinase (MMP)-9 on treatment of 4T1 cells with TGF-β (Fig. 5*A, lanes 1* and *3*). Members of the SIBLING family can interact with and activate specific MMPs (29), an activity that is critical for Bsp to promote invasion (27). We saw no effect of Bsp knockdown on the ability of TGF-β to induce pro-MMP synthesis and secretion as assayed by conventional zymography of cell supernatants, and there was no evidence for detectable activation of either MMP-2 or MMP-9 in the cell-conditioned medium or cellular lysates (Fig. 5*A* and data not shown). However, we did see a significant effect of the loss of Bsp on local matrix degradation induced by TGF-β at the cell surface, as assessed by *in situ* zymography using DQ collagens I and IV; presumably this level of MMP activation was below the detection limit of conventional zymography. Addition of TGF-β resulted in a >2-fold increase in local degradation of both collagens I and IV by 4T1 cells. Knockdown of Bsp significantly reduced local degradation of collagen both in the

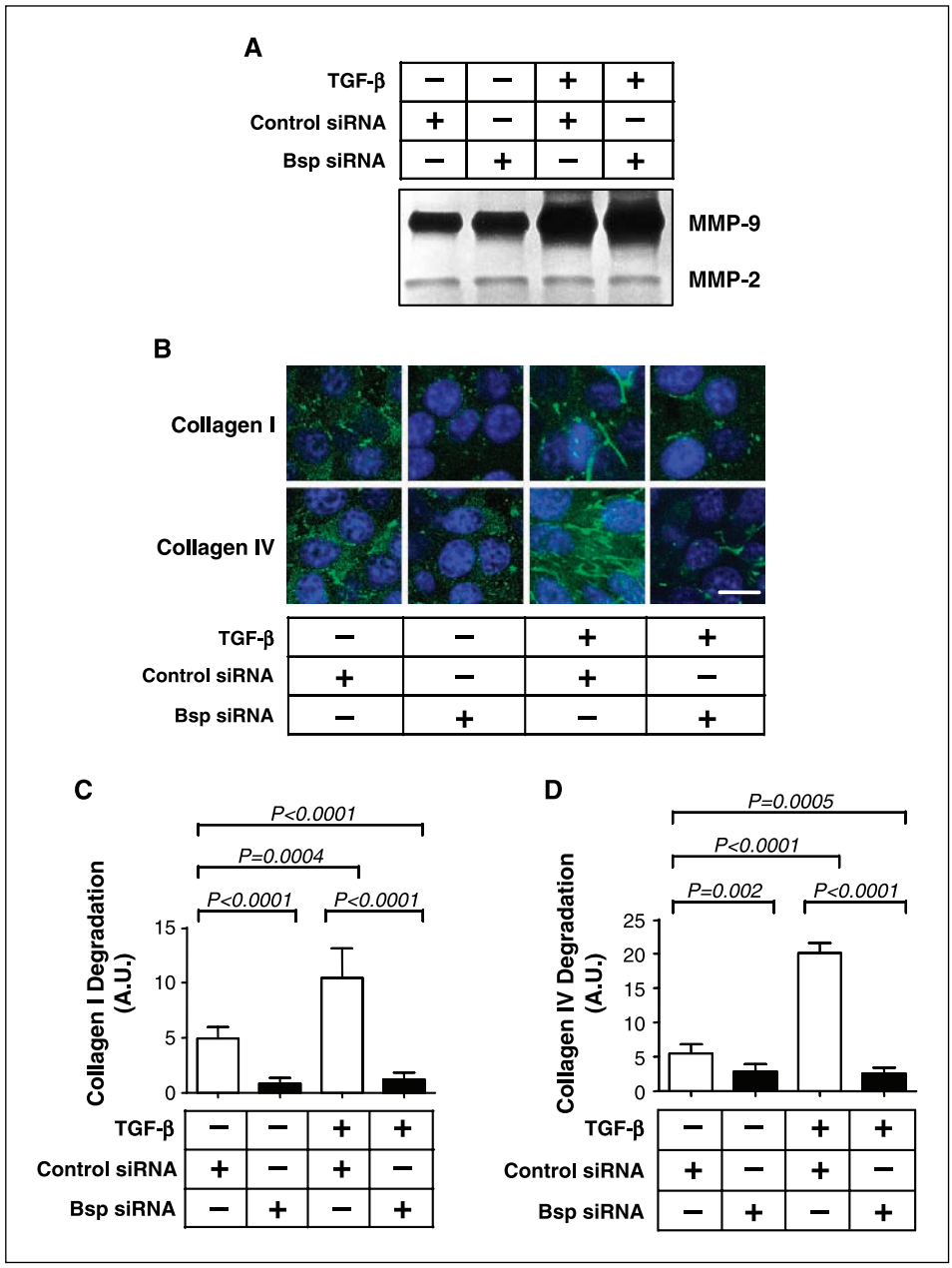

Figure 5. Bsp knockdown does not affect induction of MMPs by TGF-β but does reduce local activation of matrix degrading enzymes. *A,* MMP expression assessed by conventional zymography. The effect of Bsp knockdown on basal and TGF-β-induced MMP expression was determined by zymography on gelatin-impregnated polyacrylamide gels of conditioned medium harvested from cells, cultured on type I collagen, following treatment with 5 ng/mL TGF-β1 or vehicle for 48 hours. *B,* effect of Bsp knockdown on local matrix degradation as assessed by *in situ* zymography of cells grown within agarose impregnated with type I or type IV DQ collagen. 4T1 cells transfected with Bsp siRNA or control siRNA were treated with TGF-β1 for 48 hours and analyzed by confocal microscopy. Local matrix degradation is visualized by release of the green fluorophore. Cell nuclei are visualized by DAPI staining. Bar, 20 μm (*C* and *D*). Quantitation of extracellular matrix degradation in the *in situ* zymography assay. The intensity of green fluorescence, representing degraded collagen, was quantitated for nine random high-power fields and normalized to the number of cells within the field, determined by DAPI staining (*blue*), as detailed in Materials and Methods. *C,* type I collagen matrix; *D,* type IV collagen matrix.

basal state and following TGF-β treatment (Fig. 5*B-D*). Taken together, these results show that TGF-β-induced expression of Bsp could play a critical role in increasing invasion of the cancer cell by stimulating local extracellular matrix degradation.

Bsp protects 4T1 cells from complement-mediated cell lysis. Three members of the SIBLING family of proteins, including Bsp, can confer short-range protection of tumor cells from complement-mediated attack (19, 30). Increasing concentrations of human serum led to decreased viability of the 4T1 cells, an effect that was lost if the serum was heat treated to inactivate the complement (Fig. 6*A*). However, incubation of the cells with purified recombinant Bsp before the addition of normal human serum preserved cell viability (Fig. 6*B*). Thus, by inducing Bsp, TGF-β could promote metastasis both through enhanced invasion and through evasion of lysis by the alternative complement pathway.

Knockdown of Bsp reduces metastasis of 4T1 cells to the lung. Experimental overexpression of Bsp can promote metastasis of the MDA-MB-231 human breast cancer cell line (25) but, to date, no one has shown that endogenous Bsp is important for visceral metastasis. To separate the effects of Bsp on metastasis from any possible effects on the primary tumor, we addressed this question using the tail-vein injection (Fig. 7*A*), rather than the orthotopic implantation route, for introducing the 4T1 cells. In this format, fewer but larger metastases are formed than with orthotopic implantation, but treatment with 1D11 antibody still caused an ~2-fold reduction in the number of metastases (Fig. 7*B*). Bsp knockdown significantly reduced the number of metastatic lung nodules to an extent that was similar to that induced by treatment with 1D11 anti-TGF-β antibody (Fig. 7*C*).

Discussion

Many advanced human tumors markedly overexpress TGF-β, which allows the tumor cell to generate a more permissive stromal environment through paracrine mechanisms such as enhanced angiogenesis and reduced immune surveillance. However, due to the accumulation of genetic and epigenetic defects, in the later stages of the carcinogenic process, the tumor cell itself can also respond to TGF-β in a manner that would promote progression, by reactivating developmentally inappropriate migratory and invasive programs. Therapeutic application of TGF-β antagonists could potentially suppress metastasis by interfering with either or both of these types of mechanisms. Although it is clear that bulky TGF-β antagonists such as antibodies could readily interfere with the longer-range effects of tumor-derived TGF-β, it is less clear whether these agents would have an impact on very short-range effects of TGF-β on the tumor cell itself. To address this question, we recovered metastatic cells from the lungs of tumor-bearing mice treated with an anti-TGF-β antibody and looked for direct effects of the therapeutic intervention on the metastatic tumor cell.

Applying this approach to the 4T1 mouse model of metastatic breast cancer, we have shown for the first time that integrin-binding sialoprotein (Bsp) is an important mediator of the direct prometastatic effects of TGF-β on the tumor cell. *In vivo*, treatment with TGF-β antibody was associated with reduced expression of Bsp in lung metastases and the functional significance of this observation was confirmed when we showed that experimental knockdown of Bsp in the 4T1 cells significantly reduced their metastatic efficiency. Furthermore, Bsp and TGF-β expressions were tightly correlated with each other, as well as with metastatic ability, in a series of related breast cancer cell lines of differing metastatic potentials, suggesting that TGF-β is a key regulator of Bsp expression during progression.

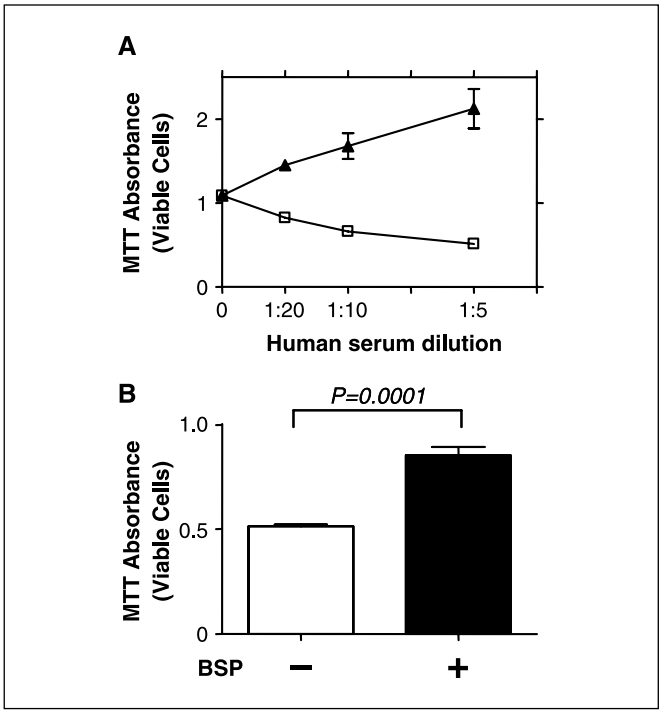

Figure 6. Bsp protects 4T1 cells from complement-mediated cell lysis. *A,* 4T1 cells are susceptible to complement-mediated cell lysis. 4T1 cells (5×10^6 cells/mL) were suspended in GVB-MgEGTA buffer and incubated with different concentrations of normal human serum, with (▲) or without (□) prior heat treatment to inactivate the complement. After 2 hours, cell viability was evaluated by colorimetric MTT assay. *B,* Bsp protects against complement-mediated lysis. 4T1 cells were incubated with purified human Bsp protein (10 μg/mL) for 10 minutes before incubation with normal human serum diluted 1:5 for the lysis assay as above. *Columns,* mean of three determinations; *bars,* SD.

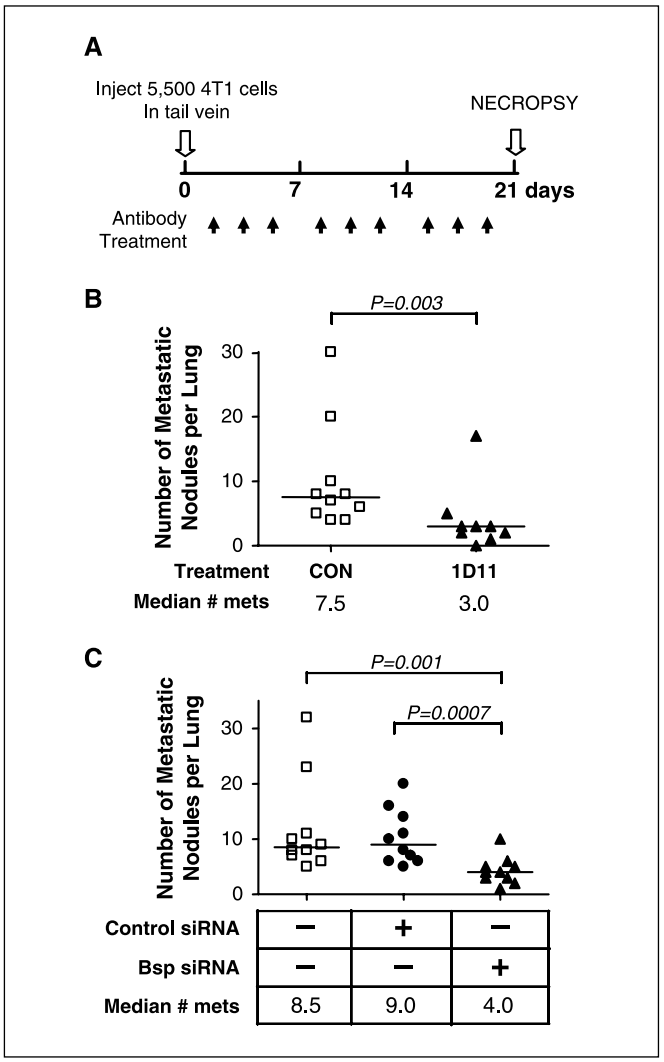

Figure 7. Knockdown of Bsp suppresses metastasis of 4T1 cells to the lung. *A,* experimental schema. 4T1 cells (5.5×10^3) were inoculated into tail vein of BALB/c mice. Where relevant, either 1D11 (5 mg/kg) or control 13C4 antibody (5 mg/kg) was administered thrice per week i.p., starting 1 day after cell inoculation. Mice were euthanized for necropsy on day 21. *B,* effect of 1D11 on metastatic efficiency. Mice were injected with 4T1 cells and then treated with 1D11 or control antibody. Grossly visible lung metastases were quantitated at necropsy range (control group, 10 mice; 1D11 group, 9 mice). *C,* effect of Bsp knockdown on metastatic efficiency. Mice were injected with parental 4T1 cells or 4T1 cells that had been transfected with either Bsp siRNA or control siRNA. Metastases were assessed as in (*B*). All experimental groups contained 10 mice. Median numbers of metastases for each group are indicated ($n = 10$ for each group).

Bsp is a member of the SIBLING family of glycoproteins that also includes osteopontin. Both Bsp and osteopontin are major constitutents of the noncollagenous matrix in skeletal tissues, where they play important roles in bone turnover (23), and both are regulated by TGF-β (26, 31). In addition to its role in bone physiology, osteopontin has recently been implicated as an important player in metastasis to multiple sites (32, 33) but Bsp is less well studied in this regard. However, a large body of clinical data shows that Bsp protein is overexpressed by many malignant tissues, including breast (34–39). Serum levels of Bsp are increased in

colon, breast, and prostate cancer patients (16), and elevated serum Bsp in primary breast cancer patients is prognostic for bone metastasis (40). Moreover, by *in silico* analysis of large clinical microarray studies of breast cancer,[6] we have found that Bsp mRNA expression in the primary tumor increases with increasing tumor grade, as has previously been shown in a smaller-scale study (39), and, furthermore, that Bsp mRNA levels correlate significantly with the presence of metastatic disease (refs. 41, 42: Supplementary Fig. S1). These correlative clinical studies are all consistent with an important role for Bsp in breast cancer metastasis. Indeed, forced overexpression of Bsp in the MDA-MB-231 human breast cancer cell line can enhance invasion and migration *in vitro* and promote metastasis *in vivo* (25, 43), and anti-Bsp antibody treatment can suppress the formation of osteolytic metastases by these cells (44). Our study is the first to show a role for endogenous Bsp in metastasis to visceral organs and to place Bsp as a downstream mediator of TGF-β in this process.

Metastasis is a multistep process, and Bsp seems to contribute to the prometastatic effects of TGF-β at more than one of these steps. Members of the SIBLING family bind to integrins on the cell surface and mediate formation of tertiary complexes with other effector molecules that then modulate cell behavior. Bsp, osteopontin, and dentin matrix protein 1 can form tertiary complexes on the cell surface with $\alpha_v\beta_3$ integrin and specific metalloproteinases, thereby enhancing MMP activation (29). Bsp specifically binds to and activates MMP-2 and was the only SIBLING that could enhance invasion in multiple cancer cell lines (27). TGF-βs are also thought to enhance invasiveness through a mechanism that is dependent on MMPs (28, 45). Here we have shown that the ability of TGF-β to promote invasion is completely dependent on the presence of Bsp. Knockdown of Bsp had no effect on the ability of TGF-β to enhance the synthesis or secretion of pro-MMPs and it did not cause detectable activation of MMPs in cell culture medium. However, it did significantly reduce basal and TGF-β stimulated activation of the MMPs locally at the cell surface, as evidenced by a reduction in the degradation of type I or type IV collagen in the immediate vicinity of the cell. Thus, the presence of Bsp is critical for TGF-β to cause local matrix degradation, a key step for invasion and metastasis.

Bsp can also form a tertiary complex with $\alpha_v\beta_3$ integrin and complement factor H (30). Metastasizing cells, as they travel through the blood stream, are exposed to the complement system and must control complement activity on their surfaces to avoid direct lysis, opsonization, or macrophage activation. The Bsp-mediated sequestration of factor H on the tumor cell surface can inhibit the antitumor effects of the alternate complement pathway for several cell types (19) by activating factor I and inhibiting the lytic pathway of complement (30). We showed that treatment with Bsp can also protect 4T1 cells against complement-mediated cell lysis. Because Bsp must interact with the integrin before binding factor H to be effective, this action of Bsp, like its effect on invasion, is likely to be very local (30). Thus, there are at least two distinct mechanisms whereby locally increased levels of Bsp on the cell surface could mediate the promotion of metastasis by TGF-β.

Bsp and osteopontin are thought to play particularly important roles in osteotropic metastasis. In the 4T1 model, in the format in which we have used it, the predominant metastatic site is the lung and the tumor burden in the bone is relatively low (data not shown), but our data clearly show that Bsp can significantly affect the metastatic efficiency to visceral sites in this model. We had hoped that our experimental approach might lead to identification of a circulating biomarker that would aid in the selection of patients for TGF-β antibody treatment and allow monitoring of the efficacy of TGF-β antagonism. However, when we determined serum levels of Bsp in this model, these were not significantly increased by the presence of tumor (Supplementary Fig. S2). Others have found that Bsp expression is lower in visceral metastases than in skeletal ones (46) and osteolytic bone metastases are likely to liberate additional Bsp from the bone matrix, which may explain why increases in circulating Bsp in breast cancer patients are only prognostic for bone metastases (40). In preliminary experiments using intracardiac injection of 4T1 cells, we have found that 1D11 is efficacious in reducing the burden of osteolytic bone metastases,[7] and work is in progress to assess Bsp status in this experimental setting. Thus, it remains possible that TGF-β antibody treatment might affect the circulating Bsp levels for patients with bone metastases but, nevertheless, it is clear that therapeutic benefit can be obtained even in settings in which Bsp is only locally increased in the tumor. Because Bsp mRNA is elevated in metastatic compared with nonmetastatic breast cancers (Supplementary Fig. S1*B*), Bsp mRNA levels in the tumor might form a useful component of a patient stratification scheme even if circulating protein levels do not.

In summary, in this study, we have identified Bsp as a novel mediator of the prometastatic effects of TGF-β on the tumor cell. Because Bsp expression in metastases is down-regulated by systemic treatment with anti-TGF-β antibodies, local suppression of Bsp in the tumor cell may contribute to the efficacy of this therapeutic approach. In other model systems, systemic antagonism of TGF-β has been shown to enhance immune surveillance and suppress angiogenesis (7, 8, 47). Treatment with anti-TGF-β antibodies enhanced the ability of dendritic cell–based vaccines to inhibit the growth of 4T1 primary tumors (48); thus, immune mechanisms are also likely to contribute to the antimetastatic efficacy of TGF-β antagonism in the 4T1 model. Indeed, we have preliminary data suggesting that efficacy is at least partially dependent on the presence of $CD8^+$ T-cells.[8] The relative contributions of the tumor- and stroma-targeted mechanisms are likely to vary among tumor types, depending on which steps in the metastatic cascade pose the biggest barrier to efficient metastasis, and the extent to which they are TGF-β dependent. A more complete understanding of the molecular mediators of the various prometastatic effects of TGF-β should help guide the clinical application of TGF-β antagonists.

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[6] http://www.oncomine.org.

[7] J. Pinkas and S. Lonning, unpublished data.

[8] J-S. Nam and L.M. Wakefield, unpublished observations.

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